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Modeling of chromosomal epigenetic silencing processes

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Silenced genes in eukaryotes are packaged into heterochromatin. In addition to establishing a passive storage site for inactive genes in differentiated cells, silencing can play an active role in promoting cellular differentiation. Here, we describe quantitative modeling of silencing processes.

Key words: heterochromatin, cellular memory, bistability, SIR, Sum1

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The term "epigenetics" refers to the observation that cells of identical genotype can display different phenotypes. The classical characteristics of epigenetic phenomena are that they encompass a binary switch between two distinct expression states (ON and OFF) rather than a graded response, that they are heritable through mitosis (and sometimes meiosis) and that they can persist even in the absence of the initial trigger for the expression state.[1]

Historical examples of epigenesis include position-effect variegation in flies, X-chromosome inactivation in mammals and paramutation in plants, but epigenetics can be considered more widely as a means for metazoans to differentiate from a single cell to a multicellular organism with functionally distinct tissues. Epigenetic phenomena—in this broad definition—often rely on regulatory networks that contain positive feedback loops.[2] Epigenetics—in the narrow sense—specifically refers to molecular mechanisms associated with competing activities of repressor/silencing proteins with positively acting factors at the target gene, and an interesting question then is how repression can result in two states, a hallmark of binary responses, rather than in a graded response.

Many of the mechanistic insights into gene silencing have come from the study of heterochromatin-like structures in budding yeast, *Saccharomyces cerevisiae*. Here, an archetypal form of silencing is mediated by the Sir2/Sir3/Sir4 (SIR) complex which forms heterochromatin that is found at the silent mating-type loci *HML*α and *HMR*a as well as at the telomeres.[3]

The establishment of silencing is generally considered a two-step process: It includes a recruitment step, during which the Sir2/Sir4 subcomplex is recruited to silencing nucleation sites ("silencers"),[4] and a spreading step, in which histone deacetylation by the histone deacetylase (HDAC) Sir2 enhances Sir3 and SIR binding to chromatin,[5,6] such that successive rounds of deacetylation and SIR binding lead to spreading of heterochromatin along the chromatin fibre.[7] Mechanisms comparable to this sequential deacetylation and recruitment model are at play in larger eukaryotes, for instance in the binding of heterochromatin protein 1 (HP1) and Polycomb group protein complexes to chromatin that is methylated at specific sites on histone H3.[8,9] Thus, the affinity of the silencing complex for its substrate (chromatin) and its ability to spread, intimately regulate the extent of silencing.

Therefore, in addition to explaining the origins of the binary response, mathematical modeling can be employed to provide a logical framework for the extent to which heterochromatin spreads along the chromosome and suppresses gene expression.

Long-Range Silencing versus Local Repression

In the past, a conceptual distinction has been made between silencing and

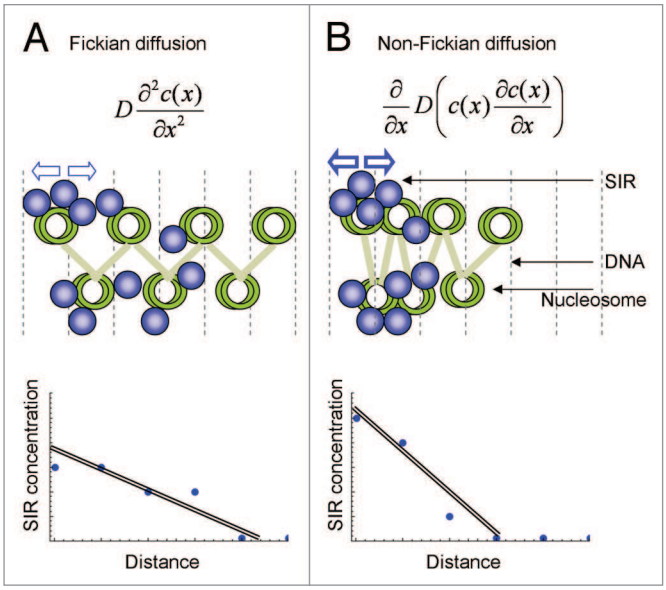

Figure 1. Models of diffusion of silencing proteins. The concentration of the SIR proteins corresponds to the number of molecules within a unit length. (A) Diffusion is Fickian when the SIR proteins perform a random walk on a stretched segment of chromatin. (B) When the chromatin condenses in the presence of SIR proteins, the SIR concentration gradient becomes steeper. Hence, the flux (flow) of SIR proteins is enhanced.

gene-specific repression. Prototypical examples include the SIR silencing and the Ssn6 co-repressor proteins.[3] While silencing is thought to be long-range and independent of the identity of the silenced gene, repression is considered a local phenomenon in which only specific promoters can be targeted for repression. The binary response as well as the mitotic inheritance were also considered specific to silencing. However, molecular work over the last years has revealed mechanistic similarities between repression and silencing, such that the distinction between the two has become more and more blurred. For example, Sum1 was initially considered a repressor protein of meiotic genes during vegetative growth in *S. cerevisiae*.[10] Yet, Sum1, similarly to SIR-mediated silencing also recruits an HDAC complex to the target genes, which in this case includes the Sir2 homolog Hst1 [10] and thus, both repression and silencing entail histone deacetylation. In line with this, the yeast *Klyveromyces lactis* contains a single Sir2/Hst1 protein that has characteristics of both *S. cerevisiae* homologs.[11] The convergence of silencing and repression is further evidenced by the fact that one of the *HM* silencers, *HML*-E, comprises a Sum1 binding site whose function becomes revealed upon mutation of other silencer elements.[12] Thus, natural *HM* silencing apparently uses a combination of silencer and repressor proteins to achieve stable silencing. In fact, Sum1 can be converted from a meiotic repressor into a silencer protein at the native *HM* loci by a single amino-acid change (Sum1-1).[13] Significantly, the conversion to silencing has been attributed to an enhanced ability of Sum1-1 to spread along chromatin.[14]

Effect of Silencing on Reporter Genes

One complicating matter with respect to the literature about silencing and repression concerns the use of different reporter genes. The detection of binary responses requires the measurement of gene expression in single cells. The green fluorescent protein (GFP) and its variants can report expression levels in single cells over a broad dynamical range. A large number of studies on silencing were performed prior to the discovery of GFP, using indirect reporters, such as the mating type-genes, *URA3* and *ADE2*.[15,16]

Most studies on *HM* silencing use the mating-type genes that naturally reside at these loci, and hence the mating ability of a cell, as a read-out. Since the mating reaction takes place in the G_1 phase of the cell cycle, this assay inherently gives a binary read-out (either a cell mates, or it does not) and it thus only measures the expression of the **a** or α genes during G_1, not throughout the cell cycle or over several cell divisions.

In contrast to the mating-type genes, the widely-used *URA3* reporter gene measures colony formation on medium lacking uracil or containing the *URA3*-counterselective agent 5-fluoroorotic acid (5-FOA), and thus by definition acts throughout the cell cycle and over multiple generations.

One reporter in *S. cerevisiae* that allows a more precise detection of gene expression in single yeast colonies is *ADE2*. Cells expressing this gene are white, whereas cells are red when it is switched off (or mutated). Thus, bistability presents a sectoring phenotype, in which a single colony consists of red and white sectors. Intermediate phenotypes also are observed, in which the colonies have a pink color that can be of varying degree, and they thus represent a graded read-out. Insertion of *ADE2* at *HMR* results in a homogeneous red colony color,[17] reflecting complete silencing, and insertion at the left arm of telomere VII causes sectoring.[16] Interestingly, using this reporter, different mutations in a single factor, PCNA (*POL30*), have been identified that cause a graded response, and others that cause sectoring.[18] It will thus be interesting to determine how PCNA can elicit both bistability and a graded response depending on how the protein function is changed.

The characterization of the quantitative features of silenced gene expression—for instance the range of silencing and binary gene expression—requires mathematical modeling (**Figs. 1 and 2**) and the precise measurement of gene expression in single cells (**Fig. 3**).[19]

Framework for Modeling

Many cellular processes are homogeneously distributed in the cell and can be viewed as well-stirred biochemical reactions. Spatially inhomogeneous reactions are also frequently encountered in a cell due to compartmentalization by

organelles or due to localization by cytoskeletal structures. Even DNA can serve as a scaffold that recruits molecules to specific parts of the genome.

In general, proteins recruited to DNA—transcription factors and regulators—have short-range effects. Thus, there is no need to consider the spatial distribution of these factors along the DNA, and it is sufficient to know the amount of transcription factors recruited to a binding site in order to predict their effect on gene expression. However, spatial modeling becomes inevitable for transcription factors that recruit effectors that spread and interact with other effectors along the DNA, as is the case for epigenetic silencing.

At the same time, DNA offers experimental means to generate quantitative data. The construction of synthetic genes with reporters that are suitable for single cell analysis, and appropriately spaced binding sites for activators and repressors/silencing proteins, allows the precise control of recruitment of regulators with little or no interference form unidentified pathways (**Fig. 3**).

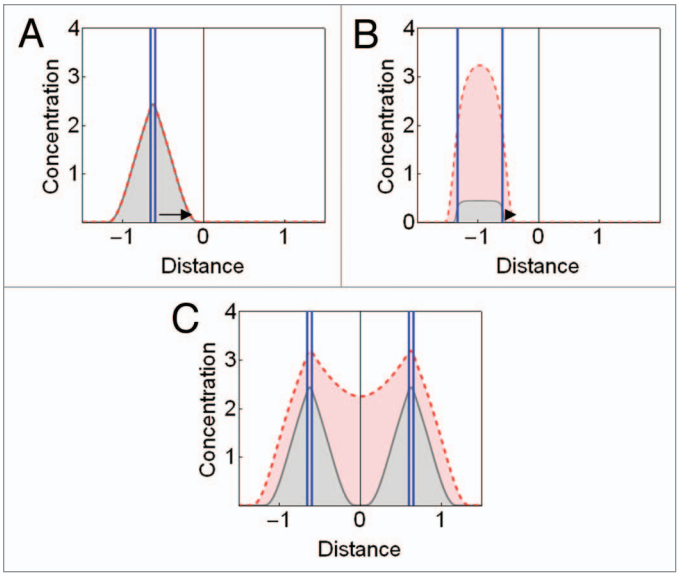

Figure 2. Calculated concentration profiles of SIR proteins (red and gray lines) as a function of nucleation sites (regions delimited by blue lines). The zero coordinate point corresponds to the transcriptional initiation site. (A) If there is a single nucleation site, the two solutions overlap (monostable system). (B) If the diffusion constant is decreased and the nucleation region is widened, two distinct solutions were obtained (bistable system).[19] However, the spread of SIR proteins beyond the nucleation segment is very limited (black arrow). (C) When two nucleation segments are in sufficient proximity, one of the solutions (red line) corresponds to the synergistic interaction of silencing sites. The system is bistable even for higher values of the diffusion coefficient.

Diffusion and Reaction of Silencing Proteins

Many proteins can bind to DNA in a weak, nonspecific way, which enables their sliding and hopping along the DNA, and this random walk can be described by Fickian diffusion (**Fig. 1A**). For Fickian diffusion, the flux (flow) of the proteins is the first derivative of their concentration gradient:

$$J = -D\frac{\partial c}{\partial x}$$

D is the diffusion coefficient, while c and x stand for the concentration and position of the proteins. The change of concentration in time is given by Fick's second law:

$$\frac{\partial c}{\partial t} = -\frac{\partial c}{\partial x}J = \frac{\partial}{\partial x}\left(D\frac{\partial c}{\partial x}\right) = D\frac{\partial^2 c}{\partial x^2}$$

Sir3 binds to both naked DNA and chromatin non-specifically,[20] which leads to condensation of chromatin and to the formation of short DNA loops by bridging neighboring DNA sequences. In this way, the platform on which the proteins perform their random walk becomes contracted in a concentration dependent manner (**Fig. 1B**). Thus, the apparent concentration gradient becomes steeper during contraction.

$$J = -D\frac{\partial c}{\partial x}$$

For the above non-Fickian diffusion, the flux increases proportionally to the concentration, which is why diffusion is nonlinear. This increased flux can be viewed as the hopping of Sir3 proteins across the DNA loops that become positioned in spatial proximity due to the contraction of the heterochromatin (**Fig. 1B**).

In the above equation, the flux approaches zero and infinity, at the corresponding concentrations. A more realistic modified version of the equation retains the linear dependence of the flux on concentration at intermediate concentration values but abolishes this dependence close to zero and at high concentration.

$$J = -\left(D_0 + D_{\max}\frac{c}{l+c}\right)\frac{\partial c}{\partial x}$$

Repressor proteins recruit HDACs, which spread along the DNA and dissociate from it. Thus, the only reactions to be modeled are association and dissociation. For silencing proteins, the sequential recruitment of the structural and enzymatic units of the SIR complex can be described by autocatalytic association kinetics. These reaction terms are then coupled to the diffusion term in the equation whose solutions represent the concentration profiles of the silencing proteins (**Fig. 2**). A source term is also included to account for the nucleation sites (silencers).

Synergy and Binary Response

A distinguishing feature of silencing that was uncovered early in the history of research on silencing is the synergistic interaction of silencers.[21] If a weak silencer is positioned either upstream or downstream of a gene, it inhibits transcription weakly or not at all.[22] However, when the upstream and downstream silencers are combined, they interact synergistically, and the inhibition is substantially stronger. The typical definition of synergy refers

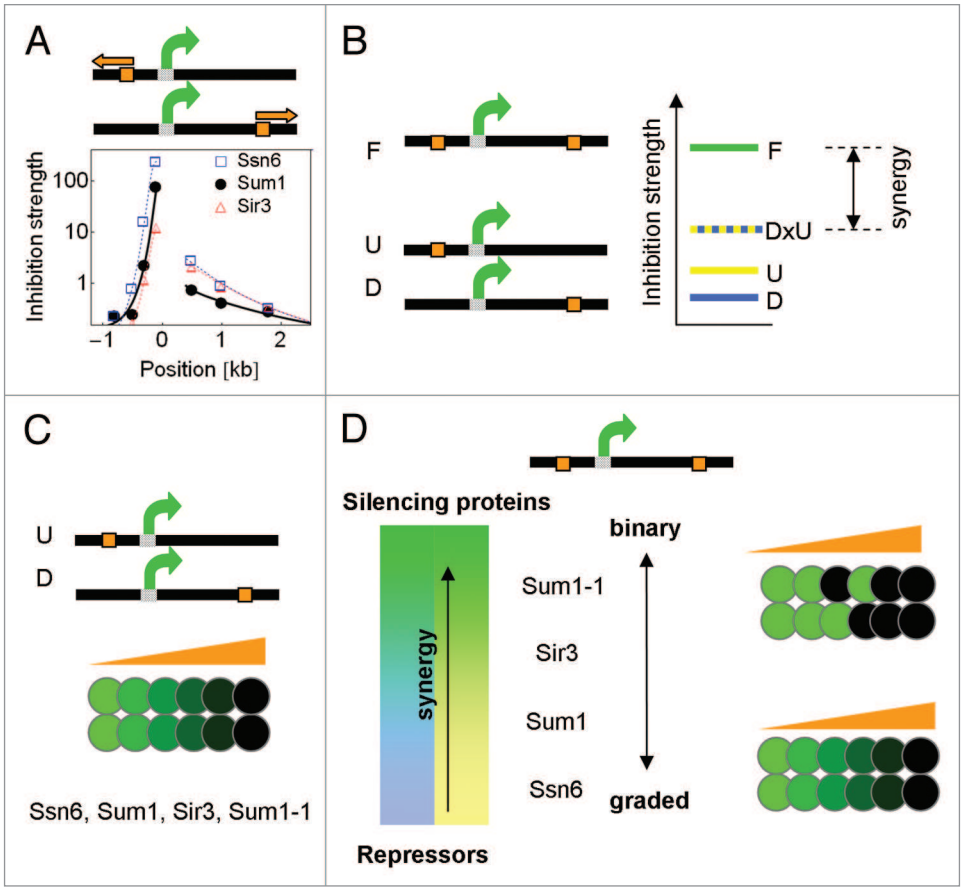

Figure 3. (A) Inhibition of gene expression, measured as a function of the distance between the binding sites for repressor/silencing proteins (Ssn6, Sum1 and Sir3) and the transcription initiation site. (B) Synergy is defined as the difference of inhibition obtained with the flanking nucleation sites (F) and the sum or product of the inhibitions obtained with the downstream (D) and upstream (U) nucleation sites. (C) When repressor/silencing proteins are recruited to a single site, they elicit a graded response in gene expression. (D) Recruitment to flanking sites results in a graded response for repressor-like proteins and in a binary response for silencing proteins.

to the case where the combination of two effects results in a combined effect that exceeds the sum of the individual effects. When the multiplicative criterion is used for synergy, the combined effect is stronger than the product of the individual effects (**Fig. 3B**). The multiplicative criterion has advantages for systems in which the two effects to be combined affect two subsequent processes. Experimental evidence indicates that repressors bound to sites upstream or downstream of the reporter gene act on distinct, possibly subsequent processes. In particular, repression from upstream sites is more sensitive to mutations in HDACs, whereas repression from downstream sites is sensitive to mutations in the mediator complex.[23]

Synergy is a common phenomenon in networks that drive cellular differentiation. Therefore, modeling is expected to link synergy to binary gene expression.

When diffusion is coupled to the autocatalytic association of silencing proteins, bistability arises only when the silencing is nucleated at two sites that flank the gene. Silencing proteins are present either at low or high concentrations around the transcriptional initiation site of the reporter gene (**Fig. 2C**). Therefore, some cells will display inhibited gene expression, while others will have only minor inhibition (**Fig. 3D**). The solution with the massive accumulation of silencing proteins corresponds to the synergistic interaction between the two nucleation sites (**Fig. 2C**). Thus, this model links bistability to synergy. Bistability arises both with Fickian and non-Fickian diffusion. However, a system with non-Fickian diffusion results in a more robust bistability, detectable over a broader range of parameter values.

When silencing is nucleated at a single site, bistability does not arise (**Fig. 2A**) unless the diffusion constant is reduced to a very low value that does not permit efficient spreading of the silencing proteins (**Fig. 2B**). In the absence of spreading, the neighboring gene would only be silenced at a very short distance. Intriguingly, this scenario may reflect the silencing of single silencers at the *HM* loci that on their own can provide considerable silencing.[22]

The binary response of a silenced gene might simply reflect slow stochastic transitions between the on and off states of the gene without the need to invoke a reaction-diffusion mechanism. This can be tested by creating a strongly silenced gene such that silencing is nucleated at a single site. If this gene is exposed to an activator, the slow stochastic transition between the off and on states would result in binary response.[24] A strong, non-synergistic silencing can be attained by a single nucleation site when it is positioned very close

to the transcriptional initiation site, resulting in a 10- to 100-fold inhibition of gene expression (**Fig. 3A**). However, bimodal expression has not been detected (**Fig. 3C**). Thus, the binary response is likely to reflect true synergistic interaction of two nucleation sites mediated by a reaction-diffusion process of silencing proteins.

Range of the Inhibitory Effect

In the context of the endogenous telomeric silencing sites, silencing also has a rather short range.[25] In semi-synthetic gene expression systems, the range of silencing was similarly short and was comparable to that of repressor proteins (**Fig. 3A**).[23] The inhibitory effect decays precipitously within a few hundred base pairs (**Fig. 3A**).

Some of the controversy over the long-range effects of silencing may be of semantic origin. The long-range effect was discovered in the context of the mating-type silencing, where two silencers interact synergistically over long distances.[26,27] Depending on the strength of the silencer, single silencers do not even repress transcription in several experimental systems.[22] Thus, a more precise term for silencing is the capability of interacting at a distance, rather than acting at a distance.

The accurate modeling of the distance dependence of repression and silencing requires a detailed mechanism for these processes. Often, multiple molecular mechanisms combine that affect the distance dependence. For example, repression is coupled to transcription of a non-coding RNA in the yeast GAL1/10 gene.[28] In such cases, models of silencing and transcriptional interference have to be combined.[19,29] The mating-type silencers themselves act both as initiators of DNA replication and as recruitment sites of SIR proteins. Thus, the complete modeling of the effect of silencers will have to merge the effects of the SIR proteins (**Figs. 2 and 3**) with replication, and other processes triggered by the silencers that affect gene expression.

Conclusions and Outlook

Current experimental evidence and modeling results suggest that repressor and silencing proteins can be characterized with respect to several properties, such as distance dependence and synergy. Most repressors and silencers examined so far have a rather uniform range of action (**Fig. 3A**). On the other hand, synergy in the interaction between upstream and downstream sites varies over a broader range (**Fig. 3D**).

The evaluation of synergy requires the measurement of gene expression over a broad range of activator and repressor binding. When the activator binding is strong, or the silencing nucleation sites are very close to the transcriptional start sites, silencing is invariably weak or strong with both single silencer and two flanking silencers, respectively. Thus, synergy will go unnoticed. Furthermore, hidden protosilencers in the genome may interact synergistically with a silencing site to be studied and, misleadingly, strong silencing thus may be attributed to the single silencer.[30] Taking the above into account may reconcile a number of apparent contradictions in the literature.

Proteins that previously classified as silencing proteins (Sir3, Sum1-1) display strong synergy, whereas classical repressor proteins (Ssn6) display weak synergy. Some proteins have intermediate values of synergy (Sum1). Thus, the boundary between silencing and repression proteins is not sharp; the degree of synergy rather forms a continuum. Silencing/repressor proteins with synergy display variegated gene expression.

It will be of interest to see whether the above system of classification can be applied for the repressor and epigenetic silencing phenomena in all organisms.

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